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for (TNF)^{12,34-41}. This factor causes necrosis *in vivo* and *in vitro*; it is also active in causing lysis of murine L-929 cells *in vitro*^{12,39}. TNF activity on L-929 cells is not inhibited by neutralizing antibodies specific for lymphotoxin¹³, however, it appears to have distinct biochemical properties^{39,40}. Furthermore, the lymphotoxin cDNA probe failed to hybridize on Northern blots to mRNA from induced macrophages producing cytolytic activity (Fig. 2). The isolation of a TNF DNA clone further demonstrates that lymphotoxin and TNF are distinct molecules (see accompanying article⁴²).

A cytolytic factor derived from B-cell lines which displays *in vivo* and *in vitro* antitumour activity has been described⁴³; this has been designated 'tumour necrosis factor' based on its activity in the MethA sarcoma assay. This activity probably results from lymphotoxin, however, because it has similar biological activities, biochemical properties and is made by the cell line PM1-1788) used in this study for the purification of lymphotoxin. Natural killer cells also can be induced to secrete an antitumour factor^{44,45}. The lymphotoxin gene probe and lymphotoxin-specific antibodies will be useful in determining the

relationship of this natural killer cytotoxic factor to lymphotoxin.

Lymphotoxin has been reported to act synergistically with α -interferon⁴⁶ and γ -interferon^{12,47} *in vitro* and *in vivo*. The potent antitumour activity of γ -interferon and lymphotoxin in natural preparations may be a result of the synergistic activity when both lymphokines are present¹³. The ready availability of lymphotoxin produced via recombinant methods will aid the biological characterization of this antitumour lymphokine. It will also help to define the antitumour mechanism of lymphotoxin, as well as its role *in vivo* in the regulation of the immune system and its interaction with other lymphokines.

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Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin

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Human tumour necrosis factor has about 30% homology in its amino acid sequence with lymphotoxin, a lymphokine that has similar biological properties. Recombinant tumour necrosis factor can be obtained by expression of its complementary DNA in *Escherichia coli* and induces the haemorrhagic necrosis of transplanted methylcholanthrene-induced sarcomas syngeneic mice.

Tumour necrosis factor (TNF) has been associated with *in vivo* and *in vitro* killing of tumour cells. This activity was covered originally in the sera of mice and rabbits injected with *Mycobacterium bovis* strain bacillus Calmette-Guérin (BCG) or other immunostimulatory agents, and subsequently

with endotoxin^{1,2}. Serum from such animals causes haemorrhagic necrosis and in some cases complete regression of certain transplanted tumours in mice^{1,2}. TNF-like activity has also been detected in the media of BCG/endotoxin-induced monocyte cultures (reviewed in ref. 2) and mitogen-stimulated peripheral

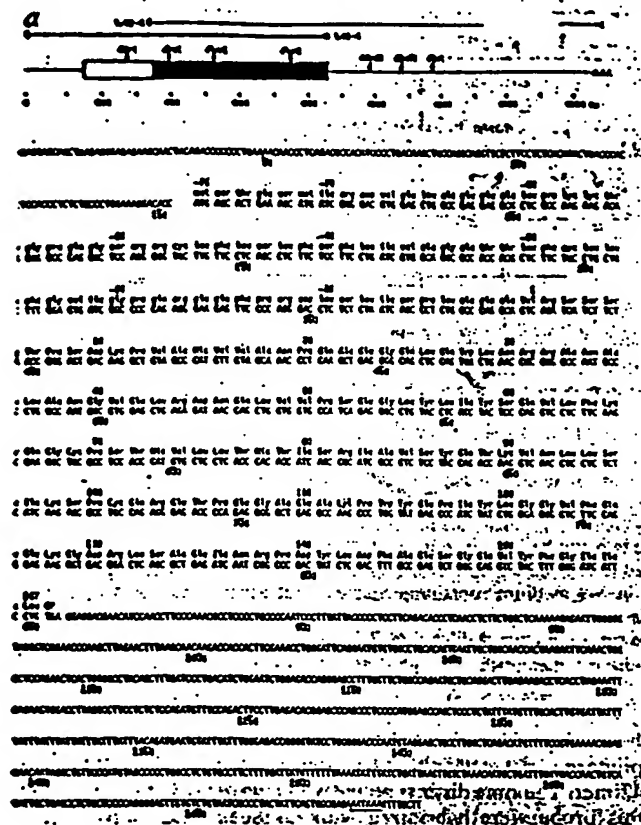


Fig. 1 TNF cDNA sequences and predicted amino acid sequence. **a**, Schematic representation of human TNF cDNA clones. Overlapping clones A2-4 and A16-4 used in sequence determination and a schematic diagram of the complete cDNA structure are shown. Line, untranslated sequences; boxes, coding sequences; white portion, sequences encoding the signal peptide; shaded regions code for mature TNF. The black box on the 3' end of clone A16-4 indicates that this clone was obtained by specific priming. **b**, Nucleotide sequence and deduced amino acid sequence of human TNF cDNA. Numbers above each line refer to amino acid positions and numbers below each line refer to nucleotide positions. The amino acid labelled 1 represents the first amino acid of mature TNF¹⁴. The 76 amino acids preceding this position are indicated by lower case lettering. Sequence underlined indicates the polyadenylation recognition site. **Methods:** **a**, Total RNA was extracted²⁵ from HL-60 cultures 4 h after PMA induction and poly(A)-containing RNA was purified on oligo(dT)-cellulose²⁶. Double-stranded cDNA was prepared by oligo(dT) priming²⁷ using 7.5 µg mRNA and fractionated on a 6% polyacrylamide gel. 700 ng cDNA >600 bp was recovered by electroelution. Synthetic EcoRI adaptor²⁸ were ligated to 20 ng cDNA before ligating into λgt10 (ref. 26). 200,000 cDNA clones were obtained. The same conditions were used to prepare a specifically primed cDNA library of 200,000 clones using as primer the hexanucleotide dTGGATGTTGCTCTCC (complementary to nucleotides 855–870). Plaque screening²⁹, P-radiolabelling of synthetic 42-mer probe³⁰ and hybridizations³¹ were performed. **b**, DNA sequencing was performed by the dideoxynucleotide chain termination procedure³². The cDNA insert of A2-4 consists of nucleotides 337–1643 and the cDNA insert of A16-4 consists of nucleotides 1–870.

blood leukocytes (PBLs)³. TNF activity is cytolytic or cytostatic against many transformed cell lines *in vitro* without obvious species specificity, yet has no known effect on normal mouse embryo fibroblasts or non-transformed cell lines^{1,2,4}. Activated macrophages may constitute the major cellular origin of TNF^{1,3,9,10}, providing an important criterion for distinguishing this factor from the lymphoid cell-derived cytotoxin, lymphotoxin¹¹. The primary structure of lymphotoxin was determined recently by protein sequencing¹² and complementary DNA cloning (see accompanying article¹³).

Table 1 Human TNF production by cell populations and cell lines

Cell source	Inducing agent(s)	Cytotoxic activity (U ml ⁻¹)	
		TNF	Lymphotoxin
Unfractionated PBLs	None	<8	<8
	LPS	20	<8
	BCG	82	<8
	BCG/LPS	86	<8
	BCG/LPS/PMA	140	<8
	PMA	280	<8
PBLs (adherent cells)	SEB/Ta ₁	100	10
	SEB/Ta ₁ /PMA	1,600	200
	None	<8	<8
	BCG/LPS	350	<8
PBLs (non-adherent cells)	SEB/Ta ₁ /PMA	590	<8
	None	<8	<8
	BCG/LPS	<8	<8
HL-60	SEB/Ta ₁ /PMA	<8	350
	None	<8	<8
	PMA	380	<8
U-937	None	<8	<8
	PMA	32	<8

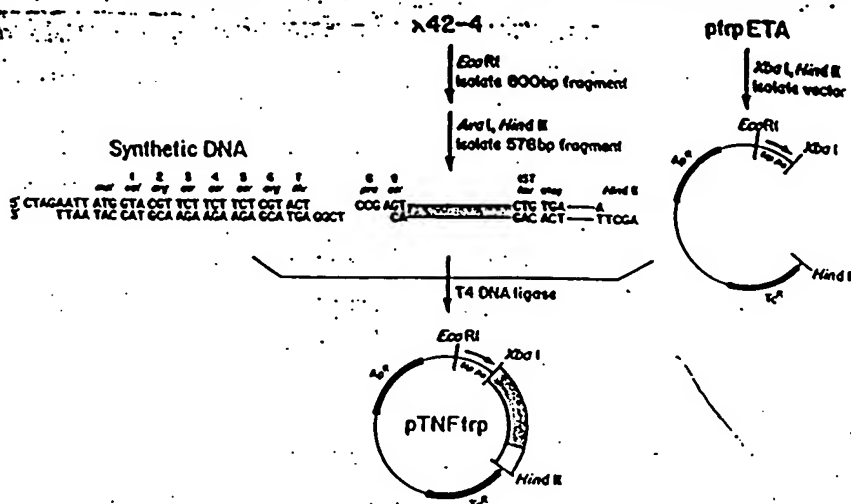
PBLs were obtained from plateletpheresis residues (Boston Red Cross) by Ficoll-Hypaque centrifugation³. Separation of PBLs into adherent and non-adherent populations was performed as described previously⁴¹. HL-60 (OCL 240) and U-937 cell lines (CRL 1593) were obtained from the American Type Culture Collection. Cells were suspended at 5×10^6 cells ml⁻¹ in RPMI 1640 media containing 10% fetal bovine serum. Cultures were induced with one or more of the following agents: 2×10^5 organisms per ml of BCG (Calbiochem-Behring), $20 \mu\text{g ml}^{-1}$ *Salmonella typhimurium* lipopolysaccharide (LPS, Sigma), $1 \mu\text{g ml}^{-1}$ staphylococcal enterotoxin B (SEB, Sigma), $1 \mu\text{g ml}^{-1}$ thymosin α_1 (Ta₁)⁴² and 10 ng ml^{-1} PMA (P-L Biochemicals). Cell-free supernatants were collected 24 h after induction except for the BCG/LPS and BCG/LPS/PMA treatments; for these two inductions a 24-h BCG stimulation was followed by an additional 24-h treatment with LPS and LPS/PMA, respectively. Samples were assayed for cytotoxic activity on mouse L-929 fibroblasts as described previously¹¹. The activities shown represent TNF-specific or lymphotoxin-specific units as determined after antibody neutralization at 4°C for 4 h before assay. The units indicated were obtained from one representative donor in the case of the PBLs and from a single experiment when cell lines were used. Rabbit anti-human TNF antiserum was prepared against partially purified TNF from PBLs (L. Svedersky and T. Brinman, unpublished results). Rabbit anti-human lymphotoxin antiserum was prepared against purified human lymphotoxin from RPMI 1788 lymphoblastoid cells¹¹.

Here we identify a cell line with monocyte-like characteristics providing a source for human TNF and its messenger RNA. cDNA clones were isolated that encode a polypeptide related structurally to lymphotoxin. This cDNA was engineered to direct the synthesis of a relative molecular mass (M_r) 17,000 protein in *E. coli* with the immunological characteristics as well as *in vitro* and *in vivo* biological properties of natural human TNF.

A human TNF-producing cell line

We isolated PBLs by Ficoll-Hypaque density centrifugation and fractionated them into adherent monocytic and non-adherent lymphocytic fractions. After stimulation with BCG and endotoxin (lipopolysaccharide, LPS), we detected an activity cytotoxic to murine L-929 cells in the culture media of unfractionated mononuclear cells and monocytes (Table 1). No cytotoxic activity was produced by the non-adherent cells following the same BCG/LPS induction procedure. The failure of rabbit anti-human lymphotoxin antibodies to neutralize the cytotoxic activity demonstrates its difference from lymphotoxin. Moreover, the results of previous *in vivo* studies using BCG/LPS induction procedures^{1,2} demonstrate that the activity can probably be attributed to TNF. Antiserum raised against partially purified PBL-produced TNF completely neutralized this activity (Table 1).

Fig. 1. Construction of a plasmid coding for the direct expression of mature human TNF in *E. coli*. The recombinant phage λ 42-4 (10 μ g) was digested with *Eco*RI and the 800-bp fragment containing the entire TNF coding region was isolated. TNF digestion with *Ava*I and *Hind*III gave a 578-bp fragment coding for amino acids 8-157. Two synthetic complementary deoxyoligonucleotides¹⁷, 5'-dCTAGAAT-TATGGTACGTTCTTCTTCGTACT and 5'-dTCGGAGTACGAGAAGAAGAACGTA-CCATAAT, were designed to code for amino acids 1-7 of TNF, preceded by an ATG translational initiation codon, and to contain an *Xba*I cohesive terminus. The choice of codons for the first six amino acids of TNF was based on *E. coli* codon usage preferences⁴⁹. An AATT sequence was incorporated upstream of the ATG to maximize expression by giving optimal spacing between the initiation codon and the *trp* leader Shine-Dalgarno sequence⁵⁰. The pBR322-derived plasmid pTrpETA⁵¹ was cleaved with *Hind*III and *Xba*I and the large fragment recovered by electroelution. The *Ava*I-*Hind*III fragment and the two synthetic deoxyoligonucleotides were inserted into the plasmid pTNFtrp expression vector to give the plasmid pTNFtrp. The methods used to assemble the fragments and verify the construction of pTNFtrp have been described previously^{19,20}. *E. coli* W3110/pTNFtrp was grown in M9 medium containing 5 μ g ml⁻¹ tetracycline to 0.2 A₅₅₀ units. Indole acrylic acid was added to a concentration of 20 μ g ml⁻¹. The cells were collected at A₅₅₀ = 1.0 and washed with cold PBS. The final cell pellet was resuspended in 1 ml PBS, sonicated on ice for 30 s and the resulting extract diluted in PBS for assay on L-929 cells¹¹.



Yields of adherent cells from peripheral blood were low and levels of TNF produced were variable and donor-dependent; therefore tested alternative induction schemes for the production of TNF from total PBLs (Table 1). An increase in cytotoxicity was observed when the PBLs were co-stimulated with *aphylococcus* enterotoxin B, desacetyl-thymosin- α , and the tumour-promoting agent 4β -phorbol 12 β -myristate 13 α -acetate (PMA). However, antibody neutralization experiments demonstrated that a significant portion of measured activity was lymphotoxin. Therefore, we screened a number of transformed cell lines of haematopoietic origin for their ability to synthesize TNF. Activity which could be neutralized by anti-TNF antiserum was detected following PMA treatment in two monocytic cell lines, HL-60, derived from a promyelocytic leukaemia¹⁴, and U-937, derived from a histiocytic lymphoma¹⁵ (Table 1). The HL-60 cell line consistently produced higher TNF titres (0.4–400 U ml⁻¹ 24 h after induction) than the U-937 cell line (100 U ml⁻¹). A time course of TNF synthesis by HL-60 cultures indicated that measurable activity was detected 2 h after PMA treatment (data not shown). Therefore the HL-60 cell line is selected for future experiments; supernatants were collected 24 h after induction for protein purification and 4 h induction was used when cells were collected for RNA isolation.

TNF cDNA clone identification

Human TNF was purified to homogeneity from filtrates of PMA-stimulated HL-60 cell cultures (see ref. 16). A single component of M_r 17,000 was observed when the purified TNF was analysed by SDS-gel electrophoresis in reducing conditions. To obtain amino acid sequence information, tryptic peptides of TNF were prepared and separated by reverse-phase HPLC. The preliminary sequence Glu-Thr-Pro-Glu-Gly-Ala-Glu-Lys-Pro-Trp-Tyr-Glu-Lys was determined for the first tryptic fragment (TD-6) analysed. A single synthetic 42-base long oligonucleotide (42-mer) which could code for this amino acid sequence was chemically synthesized¹⁷ for use as a hybridization probe. The design of the probe sequence (dGAAACCCCT-AAGGGGCTAAAGCCAGCCCTGGTATGAAAAG) was based on published human codon usage frequencies⁴⁹ and the donor bias of human γ -interferon¹⁹, tissue-type plasminogen activator²⁰ and lymphotoxin¹⁵. The general usefulness of this 'tag probe' approach has been demonstrated recently by the identification of several cloned genomic DNA sequences^{21–23} of cDNAs^{24,25}.

An oligo(dT)-primed HL-60 cDNA library of ~200,000 clones prepared in λ gt10 (ref. 26) was screened with the ³²P-labelled 42-mer. The nine distinct phage which gave positive signals with this probe were hybridized with 'induced' and 'non-induced' ³²P-labelled cDNA probes¹⁹ prepared using poly(A) mRNA obtained from 4-h PMA-treated and untreated HL-60 cultures, respectively. Seven of these recombinant phage DNAs hybridized weakly to the induced probe but did not hybridize to the uninduced probe, as expected for authentic TNF cDNAs. Restriction endonuclease mapping indicated that these seven cDNA clones were related to each other and that the phage λ 42-4 contained the longest cDNA insert.

TNF cDNA sequence

We determined the sequence of the 1,300 base pair (bp) cDNA insert of phage λ 42-4 (nucleotides 337–1,643; Fig. 1). Alignment of the cDNA sequence with the 42-mer probe sequence gave the proper reading frame of the cDNA and demonstrated that it did indeed encode TNF. Of the 14 amino acids (residues 104–117, Fig. 1) assigned to tryptic peptide TD-6 on the basis of preliminary protein sequence, 13 were correct; the only discrepancy was in the last amino acid (position 117) where the cDNA sequence encodes a proline residue rather than the predicted lysine. Despite this difference, the hybridization of the synthetic probe to the TNF cDNA clone was successful, as the 42-mer matched the cDNA sequence in 34 of the first 38 positions, including a stretch of 17 consecutive homologous nucleotides (nucleotides 711–727; Fig. 1).

The assignment of valine (residue 1, Fig. 1) as the first residue of mature TNF was based on NH₂-terminal protein sequence analysis of the intact molecule (Val-Arg-Ser-Ser-Ser-...)¹⁶. There are 156 amino acids encoded after this valine before an in-phase termination codon occurs. The coding region of TNF is followed by 792 nucleotides of 3' untranslated sequence containing the hexanucleotide AATAAA (position 1,630–1,635) which precedes the site of polyadenylation in most eukaryotic mRNAs²⁷.

Additional confirmation that this sequence codes for TNF was obtained by determining the amino acid sequence of nine tryptic peptides of natural HL-60 TNF and several peptides generated by digestion with *S. aureus* V8 protease and chymotrypsin¹⁶. The M_r of 17,356 calculated for the mature TNF monomer from the cDNA sequence agrees closely with the value obtained for natural TNF by SDS-polyacrylamide gel electrophoresis and amino acid composition¹⁶. These results and

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Table 2 Necrosis of MethA sarcoma in vivo

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Treatment	Necrotic response		
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No. of mice			
PBS, i.l.	0	1	9
PBS, i.p.	0	0	4
PBS, i.m.	0	0	4
<i>E. coli</i> LPS, i.l.	0	0	9
HL-60 TNF, i.l.	5	1	0
rTNF, i.l.	7	5	0
rTNF, i.p.	2	1	0
rTNF, i.m.	2	2	0

(BALB/cXCS7BL/6)F₁ female mice were injected intradermally with 5×10^5 BALB/c MethA sarcoma cells. Ten days later, the tumours (0.75 cm average diameter) were injected intralesionally (i.l., 1×10^5 U), intraperitoneally (i.p., 5×10^5 U) or intramuscularly (i.m., 5×10^5 U) with TNF in a total volume of 0.1 ml PBS. At 24 h after TNF treatment the tumours were excised, sectioned and scored for haemorrhagic necrosis by visual and histological examination as described previously¹. In the maximum response (++++) 50–75% of the tumour mass is markedly necrotic after 24 h; ++ denotes a moderate response, that is 25–50% haemorrhagic necrosis; +, a minimal response of <25% haemorrhagic necrosis; –, tumours showed no visible necrosis. Natural TNF was purified from HL-60 cultures as described elsewhere¹⁴. Recombinant TNF (rTNF) was purified from *E. coli* W3110/pTNFtrp to a purity of >95% and a specific activity of $\sim 10^6$ units mg⁻¹ (T. Bringman, unpublished results).

the absence of any potential N-glycosylation sites in the deduced amino acid sequence suggest that TNF is not a glycoprotein. These data suggest also that TNF may occur naturally in multimeric form, as the *M_r* estimated previously for human TNF ranged from 34,000–140,000 (refs 6, 28). There are two cysteine residues (positions 69 and 101) in TNF which are probably involved in a single intramolecular disulphide bond¹⁶.

The cDNA clone λ 42-4 contains the entire coding region of mature TNF but lacks a complete signal peptide coding sequence and initiation codon. To obtain the missing sequence information, a specifically-primed cDNA library was prepared (see Fig. 1 legend) and screened with the ³²P-labelled λ 42-4 cDNA insert. A cDNA clone (λ 16-4) was identified which contained an insert extending 337 bp further 5' than the λ 42-4 insert (Fig. 1).

From an analysis of the TNF cDNA sequence, it seems that TNF is synthesized initially as part of a larger precursor (pre-TNF). Starting at the 5' end of the cDNA, 125 nucleotides of non-translated sequence are followed by a methionine codon and an open reading frame of 233 amino acids. This AUG is preceded by termination codons in all three frames, suggesting that it is the initiation codon. Furthermore, the sequence context of this AUG conforms closely to the CC₃CCAUGG proposed²⁹ as a consensus sequence for eukaryotic initiator sites.

The presequence of 76 residues is most probably involved in the secretion of TNF as it is not observed on the mature TNF polypeptide and contains an unusually long hydrophobic region of 26 amino acids (residues –46 to –21). Typically, signal peptides involved in protein secretion are only 20–30 amino acids long^{30,31}. However, a signal sequence for the Rous sarcoma virus envelope glycoprotein³² is atypically long (63 residues) and contains also many charged amino acids at its amino terminus, such as pre-TNF. It is interesting to note the presence of Arg-Arg and Lys-Lys dipeptides in the first 30 amino acids of the TNF pre-sequence, as pairs of basic amino acids often serve as cleavage sites for the release of physiologically-important peptides from precursor molecules^{33–36}.

We used the ³²P-labelled λ 42-4 cDNA insert to examine TNF gene structure and mRNA size. Results from Southern³⁷ hybridizations indicate that only a single gene for TNF is present in the human genome. Northern hybridization analysis³⁸ shows that a single mRNA species ~ 18 S in size is synthesized in PMA-induced HL-60 cultures and BCG/LPS-treated macrophages isolated from human PBLs. This provides additional evidence that the same cytotxin is produced from both

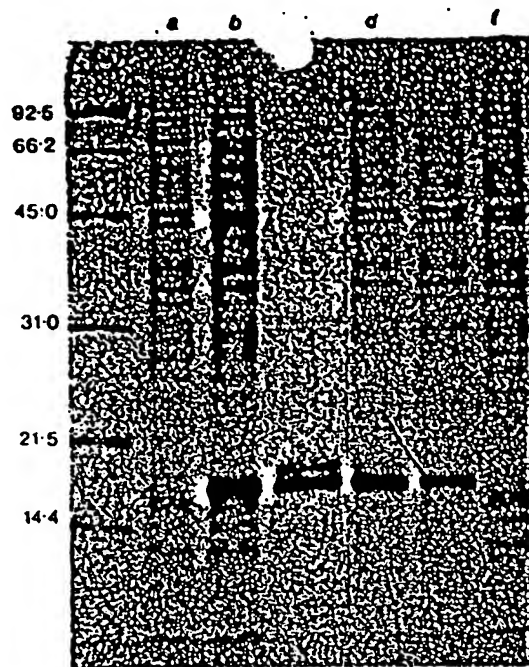


Fig. 3 SDS-polyacrylamide gel electrophoresis of human TNF synthesized in *E. coli*. *E. coli* K-12 strain W3110, transformed with pTNFtrp or pBR322, was grown in M9 medium containing $5 \mu\text{g ml}^{-1}$ tetracycline. Cells were collected, lysed in 2% SDS, 1% β -mercaptoethanol and precipitated with 10 volumes of cold acetone. Samples were electrophoresed on a 12.5% SDS-polyacrylamide slab gel using the buffer system of Maizels³² and the gel stained with Coomassie brilliant blue. The left lane contains protein *M_r* standards ($\times 10^{-3}$): phosphorylase b (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500) and lysozyme (14,500). Lanes a, f, cell lysates of *E. coli* W3110/pBR322; lanes b, c, cell lysates of *E. coli* W3110/pTNFtrp; lane d, partially purified human TNF isolated from the HL60 cell line¹⁶; lane e, mixture of the *E. coli* W3110/pTNFtrp cell lysate and the HL60-derived, purified TNF.

cell sources and suggests that the TNF cDNA sequence shown in Fig. 1 represents a nearly full-length copy of TNF mRNA. No hybridization was detected to mRNA isolated from uninduced cultures (data not shown).

TNF synthesis in *E. coli*

Proof that the cDNA described here encodes TNF requires the demonstration that it can direct the synthesis of a gene product with the properties of authentic human TNF. To allow characterization of the protein encoded by the cloned cDNA, we engineered the TNF cDNA sequence for direct expression in *E. coli* (Fig. 2). In the resulting expression plasmid, pTNFtrp, the TNF DNA sequence is under the transcriptional control of a 300-bp DNA fragment of the *E. coli* *trp* operon containing the *trp* promoter, operator and Shine-Dalgarno sequence of the *trp* leader peptide.

Total extracts of *E. coli* K-12 strain W3110 transformed with pTNFtrp contained a prominent polypeptide with an apparent *M_r* 17,000 (Fig. 3, lanes b, e). This protein is not visible in cells transformed with pBR322 (lanes a, f), strong evidence that it represents the translational product of the TNF cDNA sequence. Furthermore, this protein co-migrates with authentic TNF (lane c) isolated from the HL-60 cell line (lane d), suggesting that no significant post-translational processing of TNF occurs in the HL-60 cell line. This is unlike lymph toxin and γ -interferon, both of which occur naturally as heterogeneously glycoproteins as a consequence of N-terminal¹² and C-terminal³⁹ proteolysis, respectively.

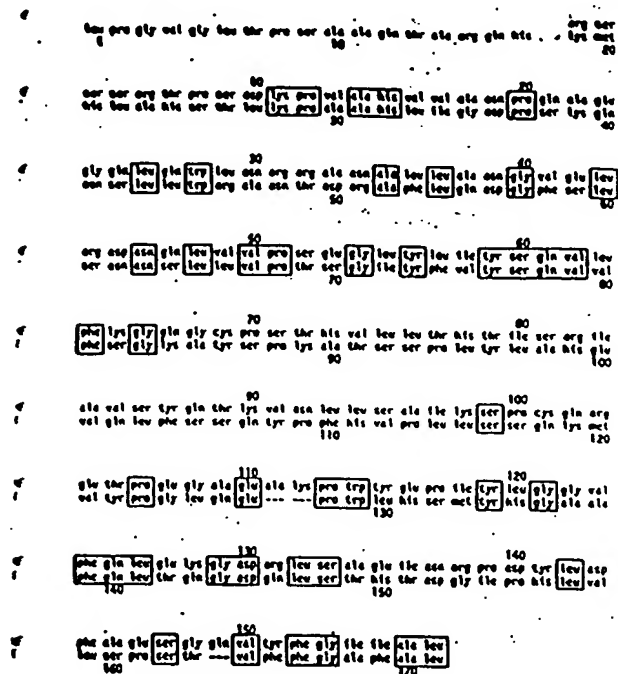


Fig. 4 Comparison of the amino acid sequence of human TNF with human lymphotoxin^{12,13}. The sequences have been aligned to give maximal homology by introducing two gaps (indicated by dashed lines) into the lymphotoxin sequence. Identical amino acids are boxed. The numbers above each row (1-157) and below each row (1-171) indicate the amino acids of mature TNF and lymphotoxin (LT), respectively.

Verification of the bacterial production of biologically-active IF was obtained by assaying extracts of *E. coli* 1110/pTNFtrp for cytolytic activity in the murine L-929 fibroblast assay¹¹. Approximately 300,000 units of activity were detected per ml of culture at $A_{550}=1$, whereas no activity was observed in *E. coli* W3110/pBR322 controls. This corresponds to ~3 mg of active TNF per l ($A_{550}=1$) or about 300,000 molecules of active TNF per cell if a specific activity of 10^6 its mg^{-1} (ref. 16) is assumed. The activity was neutralized by serum prepared against partially purified PBL-derived TNF, but was not neutralized by preimmune serum or rabbit anti-human lymphotoxin antibodies (data not shown).

in vivo necrosis activity

IF is generally defined as a cytotoxin released by BCG/LPS-activated macrophages which induces the haemorrhagic necrosis methylcholanthrene-induced (MethA) sarcomas in BALB/c mice^{1,2}. Therefore, we examined recombinant human TNF purified from *E. coli* and natural human TNF from PMA-treated HL-60 cultures for *in vivo* tumour necrosis activity in the MethA assay¹. Both recombinant and natural TNF samples showed significant necrotic responses, regardless of whether the IF was injected intrasoleally or systemically (Table 2). Minimal or no necrosis of the MethA sarcoma tumours was observed in mice injected with either phosphate-buffered saline (pH 7.4) or 100 μg *E. coli* LPS. These results, taken with the antibody neutralization and Northern hybridization data, provide further evidence that the cytotoxin described here is human IF.

Homology to lymphotoxin

It is known *in vivo* and *in vitro* biological activities of TNF and lymphotoxin are very similar^{2,3,13}. TNF and lymphotoxin are known to be antigenically distinct molecules. It has thus been common to distinguish these two lymphokines on the basis of the cell populations responsible for their synthesis. We have compared the amino acid sequences of human TNF and

lymphotoxin to determine whether similarities in their biological properties might be attributed to common structural features (Fig. 4). By introducing two gaps, the lymphotoxin sequence can be aligned with the TNF sequence so that distinct homologies are apparent; we find 44 of the 157 TNF residues (28%) are identical to corresponding lymphotoxin amino acids with many of the remaining differences between the two polypeptides resulting from conservative amino acid changes. The nucleotide homology over this coding region is 46% (data not shown). Two particularly conserved regions occur at amino acids 35-66 and 110-133 (TNF numbering) where 50% of the residues (28 of 56) are identical for TNF and lymphotoxin. The hydrophobic carboxy-termini of the two molecules are also significantly conserved. It is probable that the conserved regions are crucial to the shared cytotoxic activities of TNF and lymphotoxin, perhaps through interaction with a common receptor expressed on the surface of transformed cells. Support for this hypothesis is provided by the lack of cytotoxic activity in a truncated lymphotoxin polypeptide lacking its last 16 amino acids¹².

Lymphotoxin has 18 more NH_2 -terminal amino acids than TNF (Fig. 4), suggesting that this region is not required for cytotoxic activity. In fact, a 148 residue lymphotoxin, consisting of amino acids 24-171 of mature lymphotoxin, and having similar cytotoxic effects on L-929 cells, has been isolated from the RPMI-1788 cell line^{11,12}. It is also interesting that amino acids 67-109 of TNF are unrelated to the corresponding region of lymphotoxin; only two of 43 residues are identical. This region includes all of the amino acids spanned by the Cys 69-Cys 101 disulphide bridge of TNF. One possible role for this non-conserved region could be to position correctly the two surrounding homologous regions in a conformation essential for cytotoxic activity. Such positioning, which could be achieved by a TNF disulphide bond, may require a very different sequence of amino acids in lymphotoxin, where no disulphide bond exists. These apparently unrelated regions of TNF and lymphotoxin might specify also as yet undiscovered differences in biological activities and/or target sites between the two molecules. The availability of efficient expression systems for TNF and lymphotoxin¹⁵, in combination with the techniques of site-directed mutagenesis⁴⁰, will make it possible to address questions of this type directly.

We thank Phil Hass for growing HL-60 cells; Dr Lloyd Svedersky and Tim Bringham for preparing TNF antiserum; Mark Vasser, Parkash Jhurani and Peter Ng for deoxyoligonucleotide synthesis; Irene Figari and Refaat Shalaby for assistance with the tumour necrosis assays; Roxanne Chang and the Genentech Bioassay Group for performing *in vitro* TNF assays; and Dr Richard Harkins for helpful suggestions. G.E.N. dedicates this work to the late Jack L. Levenson.

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Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing

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A novel mammalian neuropeptide, the tachykinin substance K, is specified by a discrete genomic segment. Alternative RNA splicing generates two distinct mRNAs encoding the neuropeptide substance P alone or with substance K from a single preprotachykinin gene. Relative amounts of the mRNAs vary in different tissues, suggesting that the substance K-encoding sequence is regulated in a tissue-specific manner.

SUBSTANCE P is one of the best characterized neuropeptides in mammalian tissues; several lines of evidence suggest that it acts as a neurotransmitter or neuromodulator in primary sensory neurones¹. Substance P belongs to a family of related peptides, the tachykinins, and is thought to be the only member of this family present in mammalian tissues². Recently, we elucidated the entire primary structures of two types of bovine brain substance P precursors (α - and β -preprotachykinins) by determining their cloned cDNA sequences³. β -Preprotachykinin (β -PPT) contains not only the substance P sequence but also a novel tachykinin sequence designated substance K, whereas α -preprotachykinin (α -PPT) lacks the latter sequence, containing only substance P. The decapeptide substance K has been found independently as neurokinin α , a gut-contracting peptide in porcine spinal cord⁴. The chemically synthesized substance K peptide possesses biological activities characteristic of the tachykinin family, but is considerably more potent than substance P in some pharmacological tests^{5,6}. Substance K thus represents a second type of mammalian tachykinin which may have a physiological role different from that of substance P in mammalian organisms.

The two PPT mRNAs exhibit an interesting structural relationship. They have complete identity in their 5' and 3' sequences and differ only in the insertion/deletion of the sequence coding for the substance K region. This characteristic structural relationship poses intriguing questions about the gene organization for these two mRNAs and the regulation for the generation of the two biologically different mammalian tachykinins. Our present investigations thus concern the structural organization of the PPT gene and the distribution and regulation of the two PPT mRNAs in the nervous system and peripheral tissues. We report here that the sequence specifying the substance K region is encoded by a discrete genomic segment, and that both α - and β -PPT mRNAs arise from a single gene by alternative RNA splicing events. We also present evidence indicating the tissue-specific regulation of the PPT gene for the differential generation of the two PPT mRNAs.

PPT gene organization

Genomic clones containing the bovine preprotachykinin gene were isolated from a bovine genomic library by hybridization

in situ with a bovine β -PPT cDNA probe, and all the isolated genomic DNA fragments were arranged into an approximately 36 kilobase-pair (kbp) length of a continuous genomic DNA (Fig. 1a; see Fig. 1 legend for experimental details of cloning). Nucleotide sequence analysis was performed on DNA fragments containing exons and their surrounding regions (Fig. 1b-f). Comparison of the genomic DNA sequence with the cDNA sequence enabled us to construct a structural organization of the bovine PPT gene (Fig. 1g). Intron A (403 base pairs, bp) is inserted within the segment encoding the 5'-untranslated region of the mRNA, 9-10 bp upstream from the translational initiation site. Introns B (~1.0 kbp), C (~450 bp), D (~460 bp), E (~1.4 kbp) and F (~3.6 kbp) all interrupt the protein-encoding region of the gene. The sequences at the exon-intron boundaries are consistent with the splice junction sequences observed for other genes⁷. Exons 2-7 consist of 132, 97, 45, 24, 54 and 596 bp, each encoding the protein sequence corresponding to the signal peptide, substance P, two spacer sequences, substance K, and the C-terminal sequence, respectively. It is remarkable that exon 6 precisely specifies the substance K region missing in α -PPT. Because blot-hybridization analysis of total cellular DNAs (data not shown) as well as the genomic cloning described above showed that no more than one PPT gene is present in the bovine genome, we conclude that both α - and β -PPT mRNAs are produced from a single gene as a consequence of alternative RNA splicing events.

The 5' termini of the PPT mRNAs were identified by S₁ nuclease mapping and primer extension analyses (Fig. 2). Both analyses revealed a length heterogeneity at the 5' end of the PPT transcripts. The major 5' termini of the PPT mRNAs mapped at 106-108, 110 and 111 bp upstream from the 3' end of exon 1 (Fig. 1g). Several minor mRNA species starting further upstream were also observed and these 5' termini mapped at roughly 132, 133, 137 and 146 bp upstream from the 3' end of exon 1. In support of these assignments, we found that three of the four cDNA clones isolated previously³ (clones pSP301, 302 and 306) contained the extreme 5' sequences corresponding to the major 5' ends, while the remaining one (clone pSP307) extended its 5'-terminus up to one of the minor 5' ends. Based on the assignments of the 5' termini of the PPT mRNAs, we conclude that the bovine PPT gene is ~8.4 kbp long.



Search for

1: QWHUN tumor necrosis factor alpha precursor - human

BLink, PubMed, Related Sequences, Taxonomy, OMIM

LOCUS QWHUN 233 aa PRI 04-FEB-2000
DEFINITION tumor necrosis factor alpha precursor - human.
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 superfamily: tumor necrosis factor;
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KEYWORDS cytokine; cytotoxin; glycoprotein; homotrimer; lipoprotein; lymphokine; macrophage; membrane protein; myristylation.

SOURCE human:
ORGANISM Homo sapiens
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (residues 1 to 233)
AUTHORS Pennica,D., Nedwin,G.E., Hayflick,J.S., Seeburg,P.H., Derynck,R., Palladino,M.A., Kohr,W.J., Aggarwal,B.B. and Goeddel,D.V.
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MEDLINE 85086244

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AUTHORS Aggarwal,B.B., Kohr,W.J., Hass,P.E., Möffat,B., Spencer,S.A., Henzel,W.J., Bringman,T.S., Nedwin,G.E., Goeddel,D.V. and Harkins,R.N.
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JOURNAL J. Biol. Chem. 260 (4), 2345-2354 (1985)
MEDLINE 85130974

REMARK annotation; disulfide bond
REFERENCE 3 (residues 1 to 233)
AUTHORS Wang,A.M., Creasey,A.A., Ladner,M.B., Lin,L.S., Strickler,J., Van Arsdell,J.N., Yamamoto,R. and Mark,D.F.
TITLE Molecular cloning of the complementary DNA for human tumor necrosis factor
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REFERENCE 4 (residues 1 to 233)
AUTHORS Nedwin,G.E., Naylor,S.L., Sakaguchi,A.Y., Smith,D., Jarrett-Nedwin,J., Pennica,D., Goeddel,D.V. and Gray,P.W.
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REFERENCE 5 (residues 1 to 233)
 AUTHORS Marmenout, A., Fransen, L., Tavernier, J., Van der Heyden, J., Tizard, R., Kawashima, B., Shaw, A., Johnson, M.J., Semon, D., Muller, R. et, al.
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REFERENCE 6 (residues 1 to 233)
 AUTHORS Fukuda, S., Ando, S., Sanou, O., Taniai, M., Fujii, M., Masaki, N., Nakamura, K.I., Ando, O., Torigoe, K., Sugimoto, T. and Kurimoto, M.
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 JOURNAL J. Exp. Med. 176 (4), 1053-1062 (1992)
 MEDLINE 93018820
 REMARK annotation; identification of myristylated lysines

REFERENCE 8 (residues 1 to 233)
 AUTHORS Iris, F.J.M., Bougueleret, L., Prieur, S., Caterina, D., Primas, G., Perrot, V., Jurka, J., Rodriguez-Tome, P., Claverie, J.M., Dausset, J. and Cohen, D.
 TITLE Dense Alu clustering and a potential new member of the NFkappaB family within a 90 kilobase HLA class III segment
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REFERENCE 9 (residues 1 to 233)
 AUTHORS D'Alfonso, S. and Richiardi, P.M.
 TITLE A polymorphic variation in a putative regulation box of the TNFA promoter region
 JOURNAL Immunogenetics 39 (2), 150-154 (1994)
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REFERENCE 10 (residues 1 to 233)
 AUTHORS Takakura-Yamamoto, R., Yamamoto, S., Fukuda, S. and Kurimoto, M.
 TITLE O-glycosylated species of natural human tumor-necrosis factor-alpha
 JOURNAL Eur. J. Biochem. 235 (1-2), 431-437 (1996)
 MEDLINE 96202967

COMMENT Secreted from mitogen-activated macrophages within 4-24 hours after induction, TNF-alpha can cause cytolysis of certain tumor cell lines and have an antiproliferative effect on others without detriment to normal cells. It can also act synergistically with interferon gamma to kill certain transformed cell lines.

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 Site 20
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 Region 77..233

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REMARK SEQUENCE FROM N.A.
REFERENCE 4 (residues 1 to 233)
AUTHORS Nedwin,G.E., Naylor,S.L., Sakaguchi,A.Y., Smith,D.,
Jarrett-Nedwin,J., Pennica,D., Goeddel,D.V. and Gray,P.W.
TITLE Human lymphotoxin and tumor necrosis factor genes: structure,
homology and chromosomal localization
JOURNAL Nucleic Acids Res. 13 (17), 6361-6373 (1985)
MEDLINE 86016093
REMARK SEQUENCE FROM N.A.
REFERENCE 5 (residues 1 to 233)
AUTHORS Wang,A.M., Creasey,A.A., Ladner,M.B., Lin,L.S., Strickler,J., Van
Arsdell,J.N., Yamamoto,R. and Mark,D.F.
TITLE Molecular cloning of the complementary DNA for human tumor necrosis
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JOURNAL Science 228 (4696), 149-154 (1985)
MEDLINE 85142190
REMARK SEQUENCE FROM N.A.
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Tizard,R., Kawashima,E., Shaw,A., Johnson,M.J., Semon,D.,
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JOURNAL Eur. J. Biochem. 152 (3), 515-522 (1985)
MEDLINE 86030296
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REFERENCE 7 (residues 1 to 233)
AUTHORS Iris,F.J.M., Bougueleret,L., Prieur,S., Caterina,D., Primas,G.,
Perrot,V., Jurka,J., Rodriguez-Tome,P., Claverie,J.-M., Dausset,J.
and Cohen,D.
TITLE Dense Alu clustering and a potential new member of the NF kappa B
family within a 90 kilobase HLA class III segment
JOURNAL Nat. Genet. 3 (2), 137-145 (1993)
MEDLINE 93272029
REMARK SEQUENCE FROM N.A.
REFERENCE 8 (residues 1 to 233)
AUTHORS Rowen,L., Madan,A., Qin,S., Shaffer,T., James,R., Ratcliffe,A.,
Abbasi,N., Dickhoff,R., Loretz,C., Madan,A., Dors,M., Young,J.,
Lasky,S. and Hood,L.
TITLE Direct Submission
JOURNAL Submitted (??-OCT-1999) to the EMBL/GenBank/DDBJ databases
REMARK SEQUENCE FROM N.A.
REFERENCE 9 (residues 1 to 233)
AUTHORS Jones,E.Y., Stuart,D.I. and Walker,N.P.
TITLE Structure of tumour necrosis factor
JOURNAL Nature 338 (6212), 225-228 (1989)
MEDLINE 89159409
REMARK X-RAY CRYSTALLOGRAPHY (2.9 ANGSTROMS).
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AUTHORS Jones,E.Y., Stuart,D.I. and Walker,N.P.
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REFERENCE 11 (residues 1 to 233)
AUTHORS Eck,M.J. and Sprang,S.R.
TITLE The structure of tumor necrosis factor-alpha at 2.6 A resolution.
Implications for receptor binding
JOURNAL J. Biol. Chem. 264 (29), 17595-17605 (1989)
MEDLINE 90008932
REMARK X-RAY CRYSTALLOGRAPHY (2.6 ANGSTROMS).
REFERENCE 12 (residues 1 to 233)
AUTHORS Reed,C., Fu,Z.Q., Wu,J., Xue,Y.N., Harrison,R.W., Chen,M.J. and

TITLE Weber, I.T.
 Crystal structure of TNF-alpha mutant R31D with greater affinity
 for receptor R1 compared with R2
JOURNAL Protein Eng. 10 (10), 1101-1107 (1997)
MEDLINE 98147459
REMARK X-RAY CRYSTALLOGRAPHY (2.3 ANGSTROMS) OF MUTANT ARG-107.
REFERENCE 13 (residues 1 to 233)
AUTHORS Cha, S.S., Kim, J.S., Cho, H.S., Shin, N.K., Jeong, W., Shin, H.C.,
 Kim, Y.J., Hahn, J.H. and Oh, B.H.
TITLE High resolution crystal structure of a human tumor necrosis
 factor-alpha mutant with low systemic toxicity
JOURNAL J. Biol. Chem. 273 (4), 2153-2160 (1998)
MEDLINE 98113178
REMARK X-RAY CRYSTALLOGRAPHY (1.8 ANGSTROMS) OF MUTANT M3S.
REFERENCE 14 (residues 1 to 233)
AUTHORS Van Ostade, X., Tavernier, J., Prange, T. and Fiers, W.
TITLE Localization of the active site of human tumour necrosis factor
 (hTNF) by mutational analysis
JOURNAL EMBO J. 10 (4), 827-836 (1991)
MEDLINE 91184128
REMARK MUTAGENESIS.
REFERENCE 15 (residues 1 to 233)
AUTHORS Stevenson, F.T., Bursten, S.L., Locksley, R.M. and Lovett, D.H.
TITLE Myristyl acylation of the tumor necrosis factor alpha precursor on
 specific lysine residues
JOURNAL J. Exp. Med. 176 (4), 1053-1062 (1992)
MEDLINE 93018820
REMARK MYRISTOYLATION.
COMMENT

 This SWISS-PROT entry is copyright. It is produced through a
 collaboration between the Swiss Institute of Bioinformatics and
 the EMBL outstation - the European Bioinformatics Institute.
 The original entry is available from <http://www.expasy.ch/sprot>
 and <http://www.ebi.ac.uk/sprot>

[FUNCTION] TNF IS MAINLY SECRETED BY MACROPHAGES, IT IS A CYTOKINE
 WITH A WIDE VARIETY OF FUNCTIONS: IT CAN CAUSE CYTOLYSIS OF CERTAIN
 TUMOR CELL LINES, IT IS IMPLICATED IN THE INDUCTION OF CACHEXIA, IT
 IS A POTENT PYROGEN CAUSING FEVER BY DIRECT ACTION OR BY
 STIMULATION OF INTERLEUKIN 1 SECRETION, IT CAN STIMULATE CELL
 PROLIFERATION AND INDUCE CELL DIFFERENTIATION UNDER CERTAIN
 CONDITIONS.

[SUBUNIT] HOMOTRIMER.

[SUBCELLULAR LOCATION] TYPE II MEMBRANE PROTEIN. ALSO EXISTS AS AN
 EXTRACELLULAR SOLUBLE FORM.

[PTM] THE SOLUBLE FORM DERIVES FROM THE MEMBRANE FORM BY
 PROTEOLYTIC PROCESSING.

[DISEASE] CACHEXIA ACCOMPANIES A VARIETY OF DISEASES, INCLUDING
 CANCER AND INFECTION, AND IS CHARACTERIZED BY GENERAL ILL HEALTH
 AND MALNUTRITION.

[SIMILARITY] BELONGS TO THE TUMOR NECROSIS FACTOR FAMILY.

FEATURES	Location/Qualifiers
source	1..233 /organism="Homo sapiens" /db_xref="taxon:9606"
Region	1..76 /region_name="Propeptide"
Protein	1..233 /product="TUMOR NECROSIS FACTOR PRECURSOR"
Site	1..233 19 /site_type="lipid-binding" /note="MYRISTATE."
Site	20

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/site_type="lipid-binding"
/region_name="Transmembrane region"
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/region 77..233
/region 85..86
/region 89..94
/region 99..100
/region 104..105
/site 105
/site 108
/region 109..110
/region 112..113
/site 112
/region 115..116
/region 118..119
/region 124..125
/region 132..138
/region 140..142
/bond bond(145,177)
/region 152..159
/site 160
/site 162
/region 166..174
/site 167
/region 190..191
/region 194..200
/region 207..212
/region 215..217

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Region /region_name="Helical region"
218
Site /region_name="Beta-strand region"
222
/site_type="mutagenized"
/note="E->K: BIOLOGICALLY INACTIVE."
Region 224..225
Region /region_name="Hydrogen bonded turn"
226..232
/region_name="Beta-strand region"

ORIGIN

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1 mstesmirdv elaealpkk tggpqsrrc lflslfsfli vagattlfc l hfgvigpqr
61 eefprdlsl splaqavrss srtpsdkpva hvvanpqaeg qlqwnrran allangvelr
121 dnqlvvpseg lyliysqvlf kgqgcpsthv llhttisria vsyqtkvnll saikspcgre
181 tpegaeakpw yepiylggvf qlekgdrlsa einrpdylf aesgqvfygi ial
```

//

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Peptide Synthesis Calculation Sheet:

Notebook name is "s309".

Notebook file is "S309.NBK".

Target Peptide: length = 22, MW = 2507.037

NH₂-His-Val-Leu-Leu-Thr-His-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-
Gln-Thr-Lys-Val-Asn-Leu-Leu-COOH

Resin substitution = 0.100 meq/g

Resin quantity = 3.000 g

Excess amino acid = 4.000 x

Peptide Quantity = 0.300 mMoles

Theoretical Yield = 0.752 g

Sequence checked

23-10-89

J.Y.

Starting Resin: FMOC-Leu-PepSyn-KA

*Difficult to remove attachment
to the peptide*

Peptide Re-synthesis: S323

Peptide Synthesis Calculation Sheet:

Notebook name is "s323".
Notebook file is "S323.NBK".

Target Peptide: length = 11, MW = 1238.416

NH2-Thr-Ile-Ser-Arg-Ile-Ala-Val-Ser-Tyr-Gln-Thr-COOH

Resin substitution = 0.100 meq/g
Resin quantity = 4.000 g
Excess amino acid = 4.000 x

Peptide Quantity = 0.400 mMoles
Theoretical Yield = 0.495 g

*Sequence checked
21-11-89*

Starting Resin: FMOC-Thr-PepSyn-KA

PEPTIDE TECHNOLOGY LTD

SYNTHESIS NO. 323 14 mg

N-Tyr-Ile-Ser-Arg-Ile-Ala-Val-Ser-
Tyr-Gln-Thr-GH

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